

Metabolic changes of rhizobia in legume nodules

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Bacteria have evolved a wide variety of metabolic strategies to cope with varied environments. Some are specialists and only able to survive in restricted environments; others are generalists and able to cope with diverse environmental conditions. Rhizobia (e.g. *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Azorhizobium* species) can survive and compete for nutrients in soil and the plant rhizosphere but can also form a beneficial symbiosis with legumes in a highly specialized plant cell environment. Inside the legume-root nodule, the bacteria (bacteroids) reduce dinitrogen to ammonium, which is secreted to the plant in exchange for a carbon and energy source. A new and challenging aspect of nodule physiology is that nitrogen fixation requires the cycling of amino acids between the bacteroid and plant. This review aims to summarize the metabolic plasticity of rhizobia and the importance of amino acid cycling.

Nutrition in the soil and rhizosphere

In most cases, rhizobia are heterotrophic obligate microaerophiles that can assimilate a wide range of rhizosphere carbon and nitrogen sources and feed them into the Entner-Doudoroff pathway, pentose phosphate pathway and tricarboxylic acid (TCA) cycle for much of central metabolism [1]. However, some *Bradyrhizobium* strains can use alternative electron acceptors such as nitrate and nitrite to grow anaerobically [2] and there is also a subgroup of photosynthetic rhizobia [3] in which photosynthetic electron transport makes a substantial contribution to N₂ fixation *in planta*, presumably by increasing ATP synthesis. The diverse catabolic capacity of different strains and species of rhizobia might be important in their adaptation to survival in the rhizospheres of different groups of host and non-host plants.

The metabolic diversity of rhizobia is reflected in their large, complex genomes, which range in size from 6.5 Mb (*Rhizobium etli*; www.cifn.unam.mx/retlidb/), 6.7 Mb (*Sinorhizobium meliloti*; [4]) and 7.6 Mb (*Mesorhizobium loti*; [5]) to 7.8 Mb (*Rhizobium leguminosarum*; www.sanger.ac.uk/Projects/R_leguminosarum/) and 9.1 Mb (*Bradyrhizobium japonicum*; [6]). Many genes are devoted to transport, regulation and a wide range of catabolic systems [7]. For example, rhizobia have ~170 ATP-binding cassette (ABC) transport systems (compared

with 47 in *Escherichia coli*), which enable rhizobia to access a far greater range of nutrients present at low concentrations in soil and the plant rhizosphere (oligotrophy). The identity of the substrates transported by the majority of the transporters remains unknown but it is plausible that the diversity and variety of transport systems has evolved in response to the nutritional complexity of the rhizosphere environment. Plant root exudates contain an enormous variety of different compounds [8] and they can secrete as much as 21% of fixed photosynthate [9] (or, in seedlings, even up to 40% [8]) into the rhizosphere. For example, a variety of different sugars including galacturonic acid, mannose and rhamnose are also present in exudates and in the root mucilage of legumes [10]. In addition, rhizobia can grow on a variety of unusual compounds found in the rhizosphere such as calystegines, stachydrine, homoserine, trigonelline, mimosine and rhizopines [11–16].

Little is known of what nutritional traits are important for growth and competition in the rhizobia. Indeed, the nutritional diversity of the soil and rhizosphere, where individual carbon and nitrogen sources are not dominant, suggests that an oligotrophic lifestyle might be the most successful strategy. This could explain why rhizobia do not use the strict catabolite control seen in enteric bacteria, for example [17–19], even though rhizobia have a hierarchy of preferred carbon sources.

Invading the plant

The interaction between the bacterial microsymbiont and a legume plant, which leads to the formation of nitrogen-fixing structures (nodules), starts with a specific exchange of signal compounds. Flavonoids secreted by the host plant into the rhizosphere function as inducers of the rhizobial *nod* genes [20]. *nod* gene induction results in the secretion of lipochitin oligosaccharides that are thought to bind to specific plant receptor kinases that contain LysM motifs, such as NFR1 and NFR5 in *Lotus japonicus* and LYK3 and LYK4 in *Medicago truncatula*. This initiates a complex signalling pathway involving calcium spiking in root hairs [21–23]. The result is that root hairs curl and trap the rhizobia, which then enter the root hair through tubular structures known as infection threads that are formed by the plant. The infection threads then grow into the developing nodule tissue [24].

Entry into the infection thread forms a new environment for rhizobia, which is technically difficult to investigate, explaining why little is known about rhizobial

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physiology in this environment. Gage [25] set up a model for growth of *S. meliloti* in infection threads formed in alfalfa root hair cells by using mixed populations of *S. meliloti* L5-30 marked with constitutively expressed green fluorescent protein or DsRed. A considerable number of infection threads were infected by cells of both populations, forming green and red sectors within one infection thread. This enabled real-time observation of some growth characteristics in the root hair. Growing cells formed two to three columns of sister cells within each infection thread and cell division was restricted to a region of $\sim 60 \mu\text{m}$ at the growing tip of the infection threads. Doubling times were ~ 4 h, which indicated good growth conditions in the infection thread lumen. The restriction of growth to the tip of the infection threads was recently proposed to be a result of hydrogen-peroxide-mediated solidification of the infection thread luminal matrix [26]. It is well known that oxidative stress and the bacterial response to it has a key role in nodule formation. This could be one mechanism that enables the plant to control the abortion of infection thread development and, thus, control the nodule number.

Rhizobial mutants that are deficient in catabolism of unusual carbon sources like inositol or rhamnose are often less competitive for nodule formation [27–29]. There is also evidence that the ability to catabolize rhizopines gives an advantage in nodule competition [30]. Rhizopines are inositol derivatives that are produced by bacteroids and catabolized by free-living rhizobia of the same strain, which suggests that they might give an advantage to free-living cells competing for nodulation. However, only a small fraction of strains are capable of rhizopine metabolism [31] and non-rhizobial strains were also isolated that can use rhizopines as a sole carbon source [32]. It was proposed that optimum use of nutritional compounds could be important for competition within infection threads and, thus, for rhizobial ecology and evolution [25]. Small differences in the use of compounds such as rhamnose or inositol might, therefore, determine the population that stays in the growth zone at the end of the infection thread and finally colonizes the nodule tissue. Strategies such as *in vivo* expression technology and partial microarrays have been used to try and determine what genes are expressed by bacteria in early nodule development [33,34]. A number of genes have been identified, including *nex18* and those encoding calcium-binding proteins, adenylate cyclases, secretion proteins, transcriptional regulators and genes involved in the oxidative-stress response. With the development of whole-genome microarrays, this could provide important data about the metabolism and development of rhizobia during infection thread formation and bacteroid differentiation.

Bacteroid formation

The release of bacteria into plant cells is initiated by the formation of an infection droplet. Infection droplets can form at the tip of short intracellular infection threads (e.g. in *Phaseolus* beans [35]) or, more usually, at positions in the infection threads where the cell wall gets disrupted and rhizobial cells come into direct contact with the host-cell plasma membrane [26]. The plant cell membrane

then outgrows and bacteria are taken up into the plant cell lumen by endocytosis. The newly formed structure, which consists of bacteria that are differentiating into bacteroids enclosed in a plant cell membrane, is called a symbiosome. In determinate nodules (nodules with determinate meristematic activity), individual symbiosomes fuse and/or bacteroids further divide within the symbiosome, which results in symbiosomes that typically contain several bacteroids. However, in indeterminate nodules (nodules with indeterminate meristematic activity), individual symbiosomes further divide, together with the bacteroid, which mostly results in single bacteroids within a symbiosome.

The bacterial carbon storage compound polyhydroxybutyrate (PHB) accumulates in rhizobial cells in infection threads [36,37]. This suggests that a plentiful carbon supply is available for bacteria during growth in the infection threads. However, during the formation of bacteroids in indeterminate nodules, the PHB granules are broken down (Figure 1). In indeterminate pea nodules inoculated with a *R. leguminosarum* mutant unable to make PHB, more plant starch was consumed during bacteroid development [37]. This suggests that PHB is used as a carbon and energy source for bacteroid formation and it has been proposed that this could represent a carbon burst, in which a surge of catabolic activity results in the consumption of PHB. The situation in determinate nodules could be more complex because

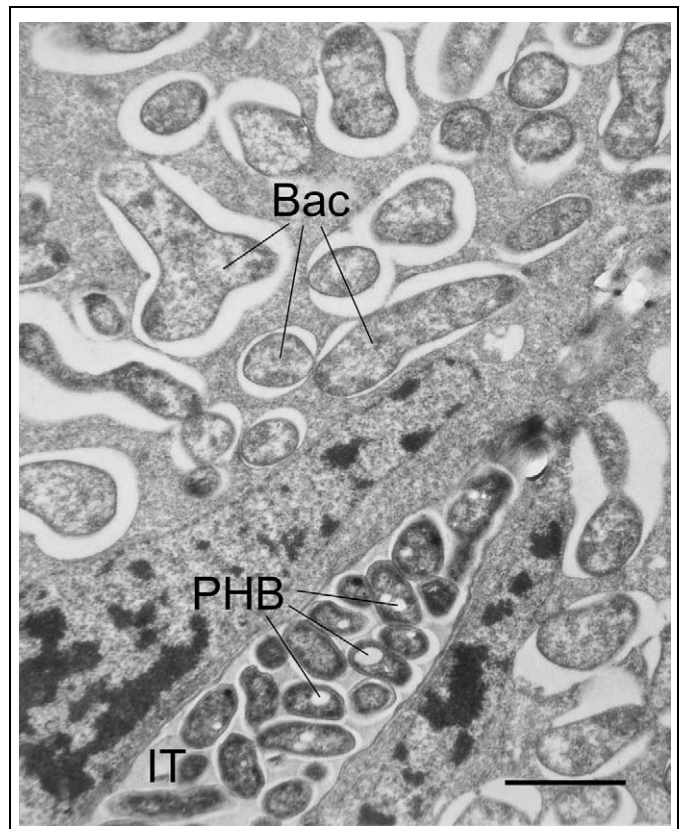


Figure 1. Electron micrograph of a pea nodule section showing an infection thread (IT) that contains rhizobia filled with PHB granules. Bacteroids (Bac) that are released into the plant cell and engulfed in a plant-derived membrane are enlarged in size and free of PHB. Scale bar = 2 μm .

mature bacteroids continue to accumulate large amounts of PHB.

Global changes in gene expression

Microarrays and proteomics have been used to examine the physiological state of mature *S. meliloti* and *M. loti* bacteroids compared with free-living cultures [38–41]. Microarray analysis of *S. meliloti* bacteroids showed that transcription of 342 genes was upregulated and transcription of 640 genes was downregulated compared with free-living cells grown on minimal medium containing succinate and NH_4^+ as carbon and nitrogen sources. Many of the downregulated genes are involved in central metabolism, which is not surprising in a non-growing cell [40]; they include genes essential for purine biosynthesis and DNA metabolism and the two main sigma factors in the cell, *rpoE1* and *sigA*. Oxygen limitation of cultured bacteria could mimic some of the changes seen in bacteroids but the overlap was only partial. As described later, O_2 -limitation is a key signal in bacteroid metabolism but there must be many others. Proteomic analysis also reveals that alfalfa bacteroids seem to be under reactive-oxygen and osmotic stress [38].

In most cases, arrays are good at detecting highly upregulated gene expression in bacteroids (e.g. *nif* and *fix* genes). There is, however, only limited agreement between proteomic and transcriptomic studies of alfalfa bacteroids [38,40]. In addition, the protein products of many genes that are apparently downregulated in microarray studies on bacteroids show high enzyme activities. Similar problems have been observed with quantitative reverse-transcriptase PCR, which suggests that the problem lies in the nature of the RNA isolated and not in the specific technique for its quantification. In particular, in pea bacteroids the pattern of rRNA and the probable ratio of rRNA to mRNA are considerably different from RNA of free-living cells (V. Ramachandran and P. Poole, unpublished).

Carbon and nitrogen exchange during symbiosis

One essential signal for the initiation of nitrogen fixation is the reduction of oxygen tension within the nodule tissue. In *S. meliloti*, O_2 tension is sensed by a haem protein sensor kinase FixL, which initiates phosphorylation of FixJ and transcription of FixK and NifA to activate expression of the *fix* (fixation) and *nif* (nitrogen fixation) genes [42]. Complex variations dependent upon FnrN and RegSR are present in other rhizobia [42–44]. In addition, key proteins such as NifA are subject to direct inactivation by high O_2 tensions in at least some rhizobia [45]. The result for all rhizobial bacteroids is the low O_2 -dependent expression of an active nitrogenase complex (iron protein and molybdenum–iron protein) and a high-affinity *cbb*₃ terminal oxidase.

Bacteroid nitrogenase catalyzes the six-electron reduction of N_2 to ammonium and has an associated reduction of 2H^+ to H_2 that uses 16–18 molecules of ATP [42]. Ammonium is secreted to the plant for incorporation into the amides glutamine and asparagine (in many legumes, and especially those with indeterminate nodules) or into ureides, which are purine derivatives

(mainly in tropical legumes with determinate nodules) [46]. There has been a long discussion about the ability of the bacteroids to assimilate the fixed ammonia themselves. The major assimilating pathway through glutamine synthetase (GS) and glutamate synthase (GOGAT) is repressed in bacteroids, and mutants in these genes are usually unaffected in nitrogen fixation [47]. Glutamate dehydrogenase activity (which results in ammonia assimilation) is not detectable in rhizobia but it has been claimed that alanine, rather than ammonium, is the sole secretion product of isolated soybean bacteroids [48]. However, this has been disputed by others [49]. If alanine were to be the sole nitrogen secretion product, instead of ammonium, then it must be synthesized by *de novo* synthesis from pyruvate and ammonium in the bacteroid and not by transamination. This is because transamination only transfers an amino group from an existing amino acid to an acceptor keto acid to form a new amino acid (see later). Alanine synthesis by alanine dehydrogenase (AldA) and secretion does occur in bacteroids but when *aldA* was mutated in either *R. leguminosarum* or *M. loti* there was no alteration in N_2 -fixation in nodules of pea (indeterminate nodules) or *Lotus corniculatus* (determinate nodules) [50,51]. Isolated pea bacteroids can make large amounts of alanine through AldA if keto acids and ammonium are accumulated in the medium [51]. This explains (at least for pea bacteroids) how large amounts of alanine can be made *in vitro* even though this process is not essential by itself *in vivo*. However, the consensus remains that, in nodules, the plant cytosol is the principal site for assimilation of ammonium into amino acids.

It has long been established that the plant provides carbon and energy sources to bacteroids in the form of dicarboxylic acids, particularly malate and succinate [46]. C_4 -dicarboxylic acids feed directly into metabolism through the TCA cycle so this suggested a simple model of bacteroid metabolism, which we have termed the classical model (Figure 2a; Figure 3). To use a C_4 -dicarboxylic acid such as malate, one molecule would normally be oxidized to oxaloacetate by malate dehydrogenase and a second molecule of malate would be oxidatively decarboxylated by malic enzyme to pyruvate. After oxidation of pyruvate to acetyl-CoA, it could be condensed with oxaloacetate to citrate, enabling the TCA cycle to proceed.

Several pieces of evidence also suggest that bacteroid metabolism might be more complex. The first of these came from exquisite (but highly complex and, therefore, often overlooked) experiments on N_2 reduction by isolated bacteroids [52]. It was shown that, whereas low concentrations of malate and succinate stimulate N_2 fixation, even modest concentrations of these dicarboxylic acids are inhibitory. Recently, an identical effect was found for pea bacteroids (E.M. Lodwig, PhD thesis, University of Reading, 2001; [51]). Soybean bacteroids seem to respond with a respiratory burst when high concentrations of dicarboxylic acid are added [52]. This could simply be a result of perturbation of the metabolism and/or respiratory chain in isolated bacteroids but an alternative is that oxidation of dicarboxylic

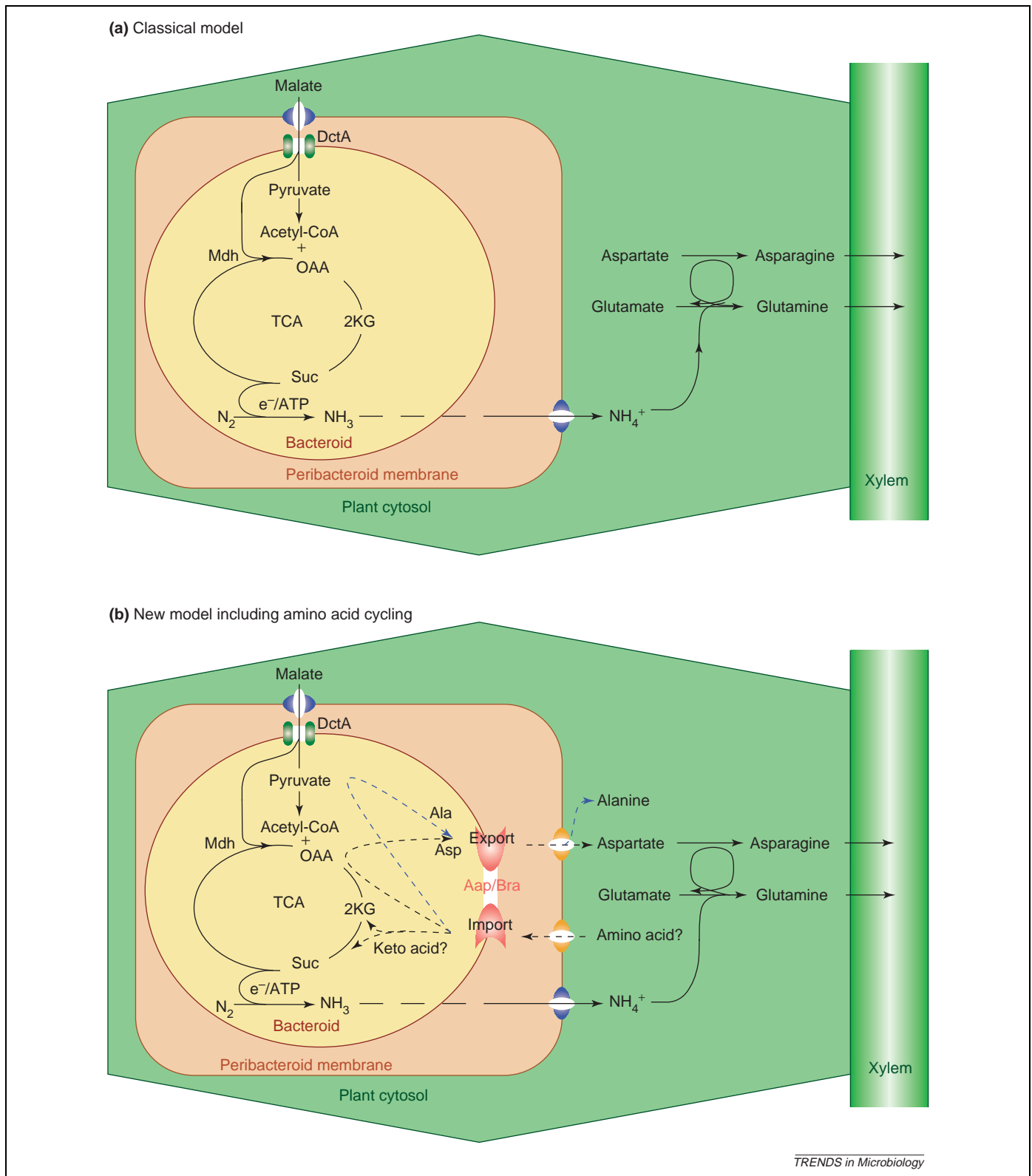


Figure 2. Models of carbon and nitrogen exchange during symbiosis with the pea plant. **(a)** Classical model showing the exchange and metabolism of the carbon source (malate, succinate or fumarate) provided by the plant and the N_2 fixed by bacteroid nitrogenase. Ammonium is then incorporated into glutamine and asparagine in the plant cytosol. **(b)** New model including amino acid cycling. In addition to the exchange of malate and ammonium, an amino acid is imported to drive transamination and secretion of alanine (Ala) or aspartate (Asp). The keto acid released during transamination is metabolized by the TCA cycle. Abbreviations: 2KG, 2-ketoglutarate; DctA, dicarboxylate uptake system; Mdh, malate dehydrogenase; OAA, oxaloacetate; Suc, succinate.

acids alone results in a highly unbalanced metabolism that is inhibitory to the TCA cycle. It should be appreciated that bacteroids are non-growing cells that must achieve a precise balance between carbon and

energy inputs and outputs. For example, an excess of carbon and reductant might simply be channelled into biosynthesis in a growing cell but would cause problems for a non-growing one.

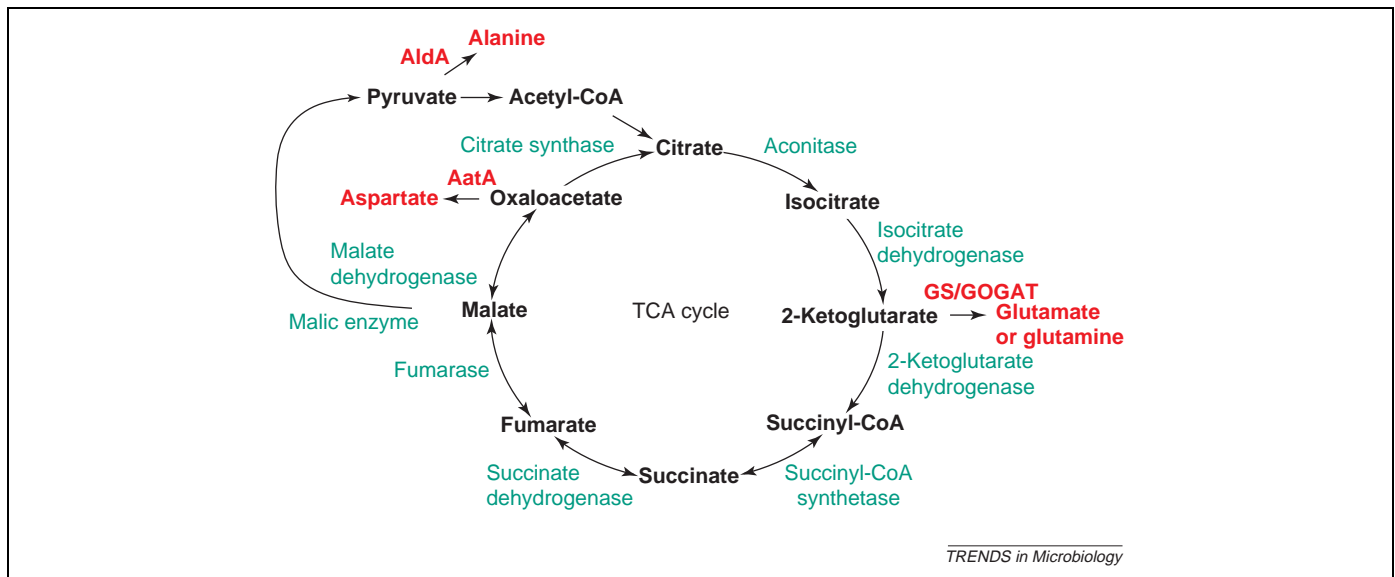


Figure 3. The TCA cycle drives the oxidation (clockwise) of its intermediates or acetyl-CoA. The enzymes that drive the cycle are indicated in green and the malic enzyme bypass to generate pyruvate is shown. Alanine, aspartate and glutamate or glutamine (red) are directly derived through ammonia assimilation or transamination from TCA cycle intermediates. Abbreviations: AatA, aspartate aminotransferase; AldA, alanine dehydrogenase; GS/GOGAT, glutamine synthetase/ glutamate synthase.

The TCA cycle

If C_4 -dicarboxylic acids are the only carbon source for bacteroids and are metabolized exclusively by the TCA cycle, it would be expected that the TCA cycle must operate fully (Figure 3). It is, therefore, highly important that aconitase mutants of *B. japonicum* still fix nitrogen normally when inoculated onto soybeans [53], the implication being that a full TCA cycle is not operating. Furthermore, although 2-ketoglutarate dehydrogenase mutants of *B. japonicum* are delayed for N_2 reduction and have lower total rates of fixation per plant, they fix N_2 at the same rate as the wild-type on a per-bacteroid basis [54]. This suggests that, although 2-ketoglutarate dehydrogenase mutants of *B. japonicum* are impaired for normal growth and development in the plant, once a bacteroid has formed it does not need a fully functioning TCA cycle. These results undermine the classical model of N_2 reduction and suggest that either a bypass of the TCA cycle must operate, such as a 2-ketoglutarate decarboxylase step [55], or that it is non-cyclic with intermediates leaving at a key branch point (see later).

A malate–aspartate shuttle?

Whereas work on regulation of TCA cycle enzymes suggested the possibility of a non-cyclic pathway in bacteroids, a number of labelling studies showed that pea, soybean or lupin bacteroids would all secrete the amino acids alanine or aspartate under N_2 -fixing conditions [51,56,57]. This was unexpected and not consistent with the simple classical model of nitrogen fixation. One highly influential suggestion from Kahn *et al.* [58] was that (by direct analogy to mitochondria) a malate–aspartate shuttle might operate in bacteroids. In the well-defined mitochondrial malate–aspartate shuttle, malate and glutamate are taken up by mitochondria. Malate is oxidized to oxaloacetate and then accepts the amino group from glutamate (releasing 2-ketoglutarate) to form aspartate. The aspartate and 2-ketoglutarate are

then exported from the mitochondria. This shuttle adds no carbon to mitochondria but does contribute reductant. Such a shuttle might operate in bacteroids to provide reductant for N_2 reduction and, possibly, even provide glutamate as a nitrogen source. However, although the labelling studies all showed alanine and aspartate secretion, keto-acid uptake and secretion rates were poor and difficult to reconcile with a malate–aspartate shuttle.

Amino acid transport

In many ways, interest in the role of amino acid secretion waned largely because there seemed to be no way to separate real secretion by isolated bacteroids from artefacts generated by their damage during isolation from nodules (Box 1). One approach taken to solve this was to mutate the amino acid uptake and secretion systems in *R. leguminosarum*. The first system to be identified and mutated was the amino acid permease (Aap), which is an ABC uptake system consisting of a solute binding protein (AapJ), two integral membrane proteins (AapQM) and an ATP-binding cassette (AapP) [59,60]. AapP almost certainly functions as a homodimer to hydrolyze ATP, driving active transport of a broad range of L-amino acids. However, it also promotes passive efflux of solutes when a substantial intracellular concentration has built up [60]. *R. leguminosarum aap* mutants are not affected in nitrogen fixation [59], however, a second ABC transporter with broad amino acid specificity (BraDEFGC) was identified and plants nodulated with double *aap-bra* mutants are severely nitrogen starved [36,61]. This is a remarkable result because whereas total $^{15}N_2$ fixation per plant is 30% of wild-type, *aap-bra* mutants retain nitrogenase activity at rates per bacteroid that equal or exceed the wild-type [36]. The implication is that ammonium assimilation in the plant is compromised. In addition, the effect of preventing amino acid movement in bacteroids clearly has a direct impact on carbon metabolism because PHB accumulates in the mutant but

Box 1. How to overcome the problems of working with isolated bacteroids

Isolated bacteroids are fragile and difficult to work with. Their outer membranes seem to be particularly delicate, and classical labelling and biochemical approaches are highly problematic. For example, the currently available methods that are used to isolate bacteroids inactivate ABC transport systems such as the crucial *Aap* and *Bra* (M. Fox and P. Poole, unpublished). Apart from the development of new and gentle ways to isolate bacteroids, there is also a need to study them with greater precision inside nodules without isolating them. High-throughput genetic screens of bacteroid genes using plants are of some use where the effects of bacteroid mutations on metabolism and nitrogen fixation can be determined. This can be difficult because either a known gene must be mutated or all genes (~6500–8000) must be mutated individually and screened on plants. Even this global screening approach is thwarted by redundancy: an example is the need to mutate both *aap* and *bra* before nitrogen fixation is affected. To study the biochemistry of bacteroids directly inside nodules, techniques such as high-resolution NMR hold great promise in studying the flux of metabolites in nodules, particularly when coupled with the use of tracers such as $^{13}\text{CO}_2$ and $^{15}\text{N}_2$. Problems with this technique include achieving sufficient resolution to determine the complex range of metabolites produced and the need to isolate nodules from the rest of the plant. When nodules are excised from legumes, an O_2 diffusion barrier is closed and nodule metabolism is effectively closed down. A complementary technique is to use metabolomic analyses in which hundreds of metabolite levels are determined both in the plant and in the bacteroid fractions of nodules that have been rapidly fractionated.

not the wild-type. This indicates that pyruvate accumulates in the *aap-bra* bacteroids and is forced into PHB rather than alanine.

Amino acid cycling

To explain these results, we suggested that an amino acid cycle must operate where an amino acid such as glutamate, or a derivative of it, is supplied by the plant to the bacteroid (Figure 2b). The bacteroid uses the amino acid to transaminate oxaloacetate or pyruvate to produce aspartate or alanine, respectively, and either or both of these amino acids are secreted. This is termed a cycle rather than a shuttle because there is no evidence that keto acids are also taken up or secreted (see earlier). Indeed, a cycle predicts that the keto acid released from the donor amino acid supplied by the plant must be metabolized by the bacteroid rather than secreted. Consistent with such a cycle, aspartate aminotransferase (*AatA*) is essential for N_2 reduction in both pea and alfalfa bacteroids [36,62]. Overall, the carbon and nitrogen metabolism of both bacteroids and plant become mutually dependent, with each feeding back to control the other.

It is clear that the identity of the amino acids that are exchanged between the plant cytosol and bacteroids needs to be established. However, this is a complex task because *Aap* and *Bra* are broad amino acid transporters that also promote efflux. To complicate matters, it is possible that more than one amino acid can be imported and exported. However, if it is assumed that one main amino acid is imported and one is exported in a simple cycle, then glutamate is a good candidate for the imported amino acid. Contradicting this, however, are studies that suggest glutamate is not easily transported across the peribacteroid membrane [63]. It also releases 2-ketoglutarate, which

would exacerbate the proposed blockage in the TCA cycle at the 2-ketoglutarate dehydrogenase step. However, if the imported amino acid transaminates pyruvate or oxaloacetate to alanine or aspartate, respectively, and the released keto acid avoids the formation of 2-ketoglutarate (some amino acids such as 4-aminobutyrate do this), then it would enable a split TCA cycle to operate. There would be no carbon build-up because the alanine and/or aspartate would be secreted. This could explain the C_4 -dicarboxylate inhibition effect because too much C_4 -dicarboxylate alone would perturb amino acid cycling and possibly force more carbon into 2-ketoglutarate. It would also explain the possible lack of a completely functioning TCA cycle because highly active aconitase and 2-ketoglutarate dehydrogenase are not needed, at least in soybean bacteroids. Finally, it would also explain the key role of transaminases in the bacteroid and the necessity for functional amino acid transport and/or efflux through the *Aap/Bra*. Despite this idea being attractive and undoubtedly ground-breaking if ever proven, it remains a hypothesis. On a final theoretical note, it is worth considering what advantages exist for the bacteroid and the plant in simply cycling one amino acid for another. Unfortunately, the correct answer to this depends on the identity of the amino acids that cycle. However, if a highly reduced amino acid such as 4-aminobutyrate is imported and a highly oxidized amino acid such as alanine is exported, then there is a large input of reductant to the bacteroid for nitrogen fixation. Finally, although *de novo* alanine synthesis through *AldA* is not essential for nitrogen fixation by itself [50,51], this does not mean that it is not important. It might have a role alongside the transamination pathway. Therefore, it still remains a considerable challenge to understand the role of transamination and amino acid secretion in bacteroids.

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